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In vitro antimicrobial activity of crude ethanol extracts and fractions of *Terminalia catappa* and *Vitex doniana*

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Abstract

Background: The spread of microorganisms resistant to some antimicrobial agents necessitates the need to search for novel and effective antimicrobial agents. In this study, the antimicrobial activity of *Terminalia catappa* Linn. (Combretaceae) and *Vitex doniana* Sweet. (Verbenaceae), two Nigerian medicinal plants used in folk medicines for the management of various ailments related to microbial infections were evaluated.

Objectives: To evaluate the antimicrobial activity of the crude ethanol extracts and fractions of the leaves and stem bark of *T. catappa* and *V. doniana*

Methodology: Four crude ethanol extracts and 16 (*n*-hexane, ethyl acetate, *n*-butanol and aqueous) fractions of leaves and stem bark of *T. catappa* and *V. doniana* were evaluated for *in vitro* antimicrobial activity against fifteen (15) strains of bacteria and fungi. The antimicrobial activity was determined in a 96-well plate using a resazurin based broth microdilution method. Two standard antimicrobial drugs ampicillin and nystatin were included as positive control.

Results: The butanol fraction of stem bark of *T. catappa* and ethanol crude extract of leaf of *V. doniana* displayed the highest antibacterial activity with similar minimum inhibitory concentration (MIC) value of 93.75 µg/mL against *S. aureus* and *B. subtilis*. Furthermore, the ethyl acetate fraction of stem bark of *T. catappa* showed the highest antifungal activity with MIC of 187.5 µg/mL against *A. sydowi*. Ampicillin had MIC of 15.6 and 31.3 µg/mL against *S. aureus* and *B. subtili*, respectively while nystatin produced MIC of 3.9 µg/mL against *A. sydowi*.

Conclusion: *Terminalia catappa* and *Vitex doniana* may serve as useful sources of plant derived antimicrobial agents.

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Keywords: *Medicinal plants; antibacterial; antifungi; resasurin broth dilution assay;*

Résumé

Contexte: La propagation de microorganismes résistants à certains agents antimicrobiens nécessite l'exigence de rechercher des agents antimicrobiens nouveaux et efficaces. Dans cette étude, l'activité antimicrobienne de Terminalia catappa Linn. (Combretaceae) et Vitex doniana Sweet. (Verbenaceae), deux plantes médicinales nigérianes utilisés dans des médicaments traditionnels pour la gestion de divers maux liés aux infections microbiennes ont été évalués.

Objectifs: Pour évaluer l'activité antimicrobienne des extraits d'éthanol brut et les fractions de feuilles et de la tige écorce de T. catappa et V. doniana

Méthodologie: Quatre extraits d'éthanol brut et 16 (n-hexane, acétate d'éthyle, n-butanol et solution aqueuse) fractions de feuilles et de l'écorce de tige T. catappa et V. doniana ont été évalués pour une activité in vitro antimicrobienne contre quinze (15) souches de bactéries et de champignons. L'activité antimicrobienne a été déterminée dans une plaque à 96-creux en utilisant une méthode de bouillon micro dilution à base de resazurin. Deux médicaments antimicrobiens standards ampicilline et nystatine ont été inclus comme témoin positif.

Résultats: La fraction de butanol de l'écorce de la tige de T. catappa et de l'extrait d'éthanol brut de feuilles de V. doniana affichaient une activité antibactérienne plus élevée avec valeur similaire de concentration minimale inhibitrice (CMI) à 93,75 µg/mL contre S. aureus et B. subtilis. En outre, la fraction d'acétate d'éthyle de l'écorce de tige de T. catappa a montré la plus haute activité antifongique avec MIC de 187,5 µg/mL contre A. sydowi. Ampicilline avait MIC de 15,6 et 31,3 µg/mL contre S. aureus et B. subtili, respectivement, tandis que la nystatine produit une MIC de 3,9 µg/mL contre A. sydowi.

Conclusion: Terminalia catappa et Vitex doniana peuvent servir de sources utiles d'origine végétale agents antimicrobiens.

Mots-clés: *plantes médicinales; antibactérienne; antifongique; dosage de dilution de bouillon resazurin;*

Introduction

The emergence and spread of micro-organisms resistant to some antimicrobial agents necessitate the need to continue the search for new and effective antimicrobial agents. Ethnomedicine is invaluable as either potential source of discovery of anti-infective compounds or as template for the synthesis of novel anti-infective molecules. *Terminalia catappa* Linn.(Combretaceae) and *Vitex doniana* Sweet. (Verbenaceae) are plants used in folk medicines for the treatment of various ailments related to microbial infections. The leaves, fruits, stem and bark of *T. catappa* are used in folk medicines for treatment of urinary infection, gastritis [1], diarrhea [2], coughs [3] and febrile illnesses [4].

Vitex doniana is a deciduous forest tree of coastal woodland, riverine and lowland forests, extending as high as upland grassland [5]. Extracts from the dried leaf of *V. doniana* is used in Nigeria to treat dizziness, diarrhoea, febrile illnesses and hypertension [6, 4]. Dried trunk is used as a chewing stick [7]. The root is also used for the treatment of fungal infections by Nigerian herbalists [8]. The extracts from *T. catappa* and *V. doniana* had been reported to show varying degrees of antimicrobial activity against some microorganisms [9-11].

However, in this study, crude extracts and fractions of the leaves and stem bark of the two plants were tested. In addition, some of the microorganisms used for antimicrobial screening of the two plants are different from previous reports. The antifungal activity of *T. catappa* and *V. doniana* against some pathogenic fungi is being reported for the first time in Nigerian ethnomedicine. Similarly, the antimicrobial assay utilized in the present study is a resazurin based broth dilution method which is more sensitive than the method previously used to evaluate the antimicrobial activity of *T. catappa* and *V. doniana.*

Materials and methods

Plant collection and authentication

Terminalia catappa and *V. doniana* were collected between August and October 2010, in Ibadan, Oyo State, Nigeria. The plant species were identified and authenticated by Mr Oluwaseun A. Osiyemi at the Forest Herbarium, Ibadan where voucher specimens were deposited under FHI 107812 and FHI 108354 for *Terminalia catappa* and *Vitex doniana*, respectively.

Extraction of plant materials

The leaf (538 g) and stem bark (1600 g) of *T. catappa* and *V. doniana* leaf (400 g) and stem bark (783 g) were powdered and each subjected to exhaustive extraction by percolation in 95% ethanol for the first 24 h. Ethanol extracts were filtered and concentrated under vacuum at 40°C using a rotary evaporator. Thereafter the marc was further percolated in 95% ethanol for another 24 h and processed as described above. The extraction process was repeated 4 times for the all plant materials, respectively. The yields of ethanol extracts were determined and extracts were stored at -20°C until needed for study.

Fractionation of ethanol extracts of leaves and stem bark of *Terminalia catappa* and *Vitex doniana*.

Crude ethanol extracts of *T. catappa* leaf (65 g) and stem bark (24 g), *V. doniana* leaf (6 g) and stem bark (18 g) were partitioned into n-hexane, ethyl acetate and n-butanol. The fractions collected after partitioning were concentrated under vacuum at 40°C. Fractions were obtained from the crude ethanol extracts and stored at -20°C till needed for study.

Microorgnisms

Strains of bacteria and fungi were procured from the Microbial Type Culture Collection at the Institute of Microbial Technology, Chandigarh, The microorganisms consisting of *Bacillus subtilis* (MTCC 121), *Micrococcus luteus* (MTCC 2470), *Staphylococcus aureus* MLS 16 (MTCC 2940), *Staphylococcus aureus* (MTCC 96), *Burkholderia cepacia* (MTCC 438), *Escherichia coli* (MTCC 43), *Klebsiella planticola* (MTCC 530), *Pseudomonas aeruginosa* (MTCC 424), *Candida albicans* (MTCC 3017), *Candida krusei* (MTCC231), *Aspergillus flavus* (MTCC 277), *A. niger* (MTCC 404), *A. parasiticus* (MTCC 2797), *A. sydowii* (MTCC 4335) and *A. ochraceous* (MTCC 4893) were used for the *in vitro* antimicrobial assay.

Antimicrobial assays

In vitro antimicrobial activity was determined using broth dilution method for the determination of minimum inhibitory concentration (MIC) [12]. Stock solution was prepared by dissolving the plant extracts, fractions and standard drugs in dimethyl sulphoxide (DMSO). Two-fold serial dilutions of plant extract/fraction concentrations (6400 µg/mL) were prepared in sterilized Mueller-Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for fungi in 96-well micro-titre plates to make seven concentrations (25 µg/mL - 6400 µg/mL). The

test plates were inoculated with freshly grown bacterial and fungal cultures adjusted to a concentration of 1.0×10^5cfu/mL with sterile normal saline. The un-inoculated sterilized medium with and without DMSO served as the control, ampicillin or nystatin served as positive control for bacteria and fungi, respectively. The plates were kept at 37°C for 24 h for bacteria, 28°C for 24 h for *C. albicans, C. krusei* and 28°C for 5 days for the rest of the fungi. After the initial incubation, five microliter (5 µL) of resazurin (5 mg/mL) was added to the test plates and plates were further incubated for 12 h. Resazurin, a non-fluorescent redox dye (blue) which is converted by viable, metabolically active cells to fluorescent resorufin (pink) was used to measure the endpoint of the assay. Minimum inhibitory concentration (MIC) was recorded as the lowest extract or fraction concentration which prevented colour change from blue to pink.

Results

The two plants, the parts of plant extracted, fractionated and percentage yields are shown in Table 1. The fractionation resulted in 16 fractions (Table 1). Crude ethanol leaves extract of *T. catappa* and *V. doniana* had higher percentage yield of 9.8 and 6.2% respectively than the stem bark of both plants. Following the fractionation of crude ethanol extract of leaves of *T. catappa* and *V. doniana* the hexane fractions had the highest percentage yield of 37.8 and 30.7% respectively. In contrast, in the fractionation of crude ethanol extract of stem bark of *T. catappa* and *V. doniana* the aqueous/methanol fraction (60.8%) and butanol fraction (38.3%) had the highest percentage yield. Minimum inhibitory concentration (MIC) of crude extracts and fractions of *T. catappa* and *V. doniana* against the strains of bacteria and fungi tested ranged from 93.75 to 3000 µg/mL and 187.5 to 3000 µg/mL, respectively (Table 2).

Ampicillin and nystatin showed MIC ranging from 7.8 to 2000 and 3.9 to 500 µg/mL, respectively (Table 2). The butanol fraction of stem bark of *T. catappa* and crude ethanol extract of leaf of *V. doniana* displayed the highest antimicrobial activity with MIC of 93.75 µg/mL against *S. aureus* or *B. subtilis*. Similarly, ethyl acetate fraction of stem bark of *T. catappa* showed the highest antifungal activity with MIC of 187.5 µg/mL against *A. sydowii* (Table 2). Plant extracts, fractions or standard drugs displaying MIC >750 µg/mL against the microorganisms were considered in active. All the crude extracts and fractions were inactive against *E.coli, P. aeruginosa, K. planticola, Candida albicans, C. krusei, A. Flavus* and *A. niger*. The extracts and fractions of the leaf and stem bark of *T. catappa* displayed greater inhibitory activity with MIC ranging from 187.5 - 750 µg/mL against the three strains of the fungi (*Aspergillus sydowii, A. ochraceus* and *A. parasiticus*) than extracts from *V. doniana* as shown in Table 2.

Discussion

In this study, the butanol fraction of stem bark of *T. catappa* displayed the highest antimicrobial activity with MIC of 93.75 µg/mL against *S. aureus* 96. The activity of the standard drug ampicillin with MIC 15.6 µg/mL against *S. aureus* 96 is higher than that of the plant extracts and fractions. Further purification of the butanol fraction may lead to isolation of compounds with higher activity. In addition, ethanol extract, hexane, ethyl acetate and butanol fractions of leaf or stem bark of *T. catappa* were more potent than the standard drug ampicillin against a resistant strain of *S. aureus* MLS16. Extracts and fractions of *T. catappa* showed varying degrees of antimicrobial activity against *B. subtilis, B. cepacea* and *M. luteus*. *Terminalia catappa* appears to have potential as antimicrobial agent in the treatment of infection cause by *S. aureus* a bacterium that is responsible for diseases such as food poisoning, septic shock, toxic shock syndrome and scalded skin syndrome. *B.cepacia* is an important

Table 1 : Percentage yield of extracts and fractions of *T. catappa* and *Vitex doniana*

Extracts/ fractions	Percentage yield (%) *T. catappa* (Leaf)	*T. catappa* (Stem bark)	Percentage yield (%) *V. doniana* (Leaf)	*V. doniana* (Stem bark)
Ethanol	9.8	6.1	6.2	1.5
Hexane	37.8	1.3	30.7	15.7
Ethyl acetate	18.4	11.0	13.5	17.0
Butanol	21.4	23.2	23.3	38.3
Aqueous/ethanol	1.1	60.8	28.1	21.2

Title 2: Antimicrobial activity of crude ethanol extracts and fractions of *Terminaliactappa* and *Vitexdoniana*

Plant extracts / fractions	Minimum Inhibitory Concentration (MIC) µg/ml														
	E. coli	*S. aureus* 96	*B. subtilis*	*B. cepacea*	*M. luteus*	*S. aureus* MLS16	*P. aeruginosa*	*K. planticola*	*A. niger*	*A. sydowii*	*A. parasiticus*	*A. ochraceous*	*A. flavus*	*C. albicans*	*C. krusei*
TCL ETOH	-	375	375	1500	375	750	-	-	-	1500	3000	3000	-	-	3000
HEX	3000	187.5	750	1500	750	750	-	-	-	750	3000	-	-	-	3000
ETOAC	3000	750	187.5	1500	375	1500	-	-	-	750	1500	3000	-	-	3000
BUT	3000	375	1500	750	375	-	-	-	-	3000	-	-	-	-	-
AQUEO	-	750	1500	-	750	1500	-	-	-	-	-	-	-	-	-
TCB ETOH	3000	187.5	750	375	375	1500	-	-	-	1500	3000	-	-	-	-
HEX	-	187.5	187.5	-	1500	1500	-	-	1500	375	750	750	-	-	-
ETOAC	3000	187.5	187.5	750	187.5	750	3000	3000	1500	187.5	750	750	1500	-	-
BUT	1500	93.75	750	187.5	187.5	750	3000	3000	-	3000	-	-	-	-	-
AQUEO	3000	187.5	750	375	187.5	-	3000	3000	-	-	-	-	-	-	-
VDL ETOH	-	1500	93.75	-	1500	-	-	-	-	3000	-	-	-	-	-
HEX	-	1500	187.5	-	1500	-	-	-	3000	3000	-	-	-	-	-
ETOAC	3000	1500	1500	-	1500	1500	-	-	-	3000	-	-	-	-	-
BUT	-	1500	1500	-	3000	-	-	-	-	-	-	-	-	-	-
AQUEO	-	3000	3000	-	3000	-	-	-	-	-	-	-	-	-	-
VDB ETOH	3000	1500	375	3000	3000	3000	-	3000	-	1500	-	-	-	-	-
HEX	-	750	375	-	1500	-	-	-	-	3000	3000	-	-	-	-
ETOAC	3000	375	1500	3000	750	-	3000	3000	-	3000	-	-	-	-	-
BUT	-	750	1500	-	1500	3000	-	-	-	3000	-	-	-	-	-
AQUEO	3000	1500	750	3000	3000	1500	-	3000	-	-	-	-	-	-	-
Standard drugs	62.5	15.6	31.3	2000	7.8	1000	2000	2000	31.3	3.9	31.3	500	62.5	7.8	15.6

Standard drugs: Ampicillin for bacteria and nystatin for fungi -: No activity . TCL – leaves of Terminalia catappa. TCB – stem bark of Terminalia catappa. VDL – leaves of Vitex doniana. VDB - stem bark of Vi. doniana. ETOH –ethanol extract. Hex – hexane fraction. ETOAc - ethyl acetate. BUT – buthanol fraction. AQUEO - Aqueous fraction

human pathogen. *Burkholderia cepacia* causes *pneumonia* in immunocompromised individuals with underlying lung disease (such as cystic fibrosis or chronic granulomatous disease [13].

The antifungal activity of the *T. catappa* observed in this study against *Aspergillus sydowii*, *A. ochraceus* and *A. parasiticus* is being reported for the first time. These fungi are of important interest; *Aspergillus sydowii*, a pathogenic fungus causes several diseases in humans while *A. ochraceus* is known to produce the toxin ochratoxin A one of the most abundant food-contaminating mycotoxins. In addition, some strains of *Aspergillus parasiticus* produce aflatoxin which is carcinogenic [14]. These plants may serve as useful sources of plant derived antifungal agents. *Terminalia catappa* is rich in flavonoids and hydrolysable tannins such as punicalagin, punicalin, terflavins A and B, tergallagin, tecatain, geraniin, granatin B, corilagin, gallic acid, corilagin, ellagic acid and rutin [15, 16]. Gallic acid, corilagin, ellagic acid and rutin possessed *in vitro* antibacterial activity [17-19].

The leaf and stem bark extracts and fractions of *V. doniana* showed lower antimicrobial activity (high MIC) in comparison with extracts and fractions of *T. catappa*. The crude ethanol extract of leaf of *V. doniana* displayed the highest antimicrobial activity with MIC of 93.75 µg/mL against *B. subtilis*. However, further fractionation of the ethanol extract resulted in fractions with lower antibacterial activity. In contrast the fractionation of ethanol extract of stem bark of *V. doniana* into solvents of different polarity resulted in improved antibacterial activity against *S. aureus* 96 and *M. luteus*. *Vitex doniana* appears inactive (high MIC values ranging from 1500 – 3000 µg/mL) against various fungi tested in the present study. Other species of *Vitex* such as *V. trifolia* showed antimicrobial activity against both Gram positive and negative bacteria [20]. In addition, one new compound vitegnoside and other five known compounds, 5'-hydroxy-3',4',3,6, 7-pentamethoxyflavone, luteolin, agnuside negundoide and *iso*-orientin, were isolated and characterized from *V. negundo*. Vitegnoside and negundoside showed promising antifungal activity against *T. mentagrophytes* and *C. neoformans* [21]. In addition, an antimicrobial compound, a labdane diterpene was also isolated from *V. rehmannii* as an inseparable epimeric mixture of 12*S*,16*S*/*R*-dihydroxy-*ent*-labda-7,13-dien-15,16-olide [22].

Conclusion

Extracts from *Terminalia catappa* and *Vitex doniana* may serve as useful sources of plant derived antimicrobial compounds. Investigations to identify the active antimicrobial compound from *Terminalia catappa* and *Vitex doniana* is in progress.

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